IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Kazuhiro FUKAE
Serial No. : 10/544,212
Filed : August 2, 2005
For : PROCESSES FOR PRODUCING SUGAR CHAIN ASPARAGINE DERIVATIVE
Art Unit : 1651
Examiner : Leon B. Lankford Jr.
Hon. Commissioner of Patents and Trademarks

DECLARATION

Sir:

I, Kazuhiro FUKAE, a Japanese national, residing at c/o OTSUKA CHEMICAL CO., LTD., 463,Kagasuno, Kawauchi-cho, Tokushima-shi, Tokushima-ken 771-0193 Japan, declare that:

1. I am an applicant of the above-identified application.

2. I have studied the Official Action dated October 5, 2007.

3. I have conducted the following experiments to demonstrate superiority of the present invention over the cited reference.

EXPERIMENTS

Example 1 of the present invention is shown below.

Example 1

One egg yolk was placed as broken into 67 ml of

ethanol (EtOH) being stirred. The mixture was stirred for about 5 hours and then filtered, followed by washing with 30 ml of EtOH. To the resulting crystals was added 83 ml of EtOH again, and the mixture was stirred overnight. The mixture was thereafter filtered, followed by washing with 30 ml of EtOH. The crystals obtained were dried, giving about 3 g of delipidated egg yolk.

(a) The delipidated egg yolk was dissolved in a phosphate buffer (7.0 in pH, 30 ml), and NaN_3 (10 mg) was added to the solution. Orientase ONS (product of Hankyu Bioindustry Co., Ltd., 1.0 g) was further added to the solution, and the mixture was allowed to stand at 50°C for about 24 hours. After the termination of the reaction was confirmed by TLC, the reaction mixture was filtered with Celite. The filtrate was concentrated and purified by gel filtration column chromatography (Sephadex G-25, 2.5 × 100 cm, H_2O). The fractions containing the desired saccharides were collected, concentrated and then freeze-dried.

(b) To the residue (about 430 mg) obtained were added Tris-hydrochloric acid · calcium chloride buffer solution (7.5 in pH, 43 ml) and NaN_3 (21 mg) to obtain a solution. Actinase E (43 mg) was added to the solution, and the mixture was allowed to stand for 24 hours while being checked for pH every 12 hours. Actinase E (21.5 mg) was added to the reaction mixture again 24 hours later, followed by a reaction again for about 48 hours while being checked for pH. After the termination of the reaction was confirmed by TLC, the reaction mixture was filtered with Celite, and

the filtrate was concentrated and purified by gel filtration column chromatography (Sephadex G-25, 2.5 × 100 cm, H_2O). The fractions containing the desired saccharides were collected, concentrated and then freeze-dried.

(c) The residue (about 120 mg) obtained was dissolved in 1.5 ml of water, and 26 mg of sodium bicarbonate was added to the solution. To the mixture was added a solution of 68 mg of Fmoc-Osu [N-(9-fluorenylmethyloxycarbonyl)-oxysuccinimide] in 2.5 ml of dimethylformamide, and the resulting mixture was reacted at room temperature for 2 hours. After the disappearance of the material was confirmed by TLC (isopropanol:1M aqueous solution of ammonium acetate=3:2), the reaction mixture was concentrated by an evaporator. To the residue were added 15 ml of water and 25 ml of diethyl ether, and the mixture was stirred for 10 minutes, followed by a separation procedure. The aqueous layer was further washed with 15 ml of diethyl ether, and thereafter concentrated and freeze-dried. The product was purified using an ODS column (Wako-Gel 100C18) for gradient elution. The fractions containing oligosaccharides were collected, concentrated and freeze-dried.

(d) The residue was purified by an HPLC fractionating column (YMC-Pack R&D ODS, D-ODS-5-A, 20 × 250 mm, AN/25 mM $AcONH_4$ buffer=20/80, 7.5 ml/min., wavelength 274 nm). A fraction of main peak eluted about 15 minutes later was collected, then concentrated and desalted on an ODS column. When freeze-dried, the product afforded about 13.3 mg of the desired disialo Fmoc oligosaccharide derivative.

Comparative Example 1

The same procedure was conducted as in Example 1 except that Actinase E in the step (b) is replaced by Orientase ONS. The desired product is obtained in an amount of 4.4 mg. Here, protease only is used and peptidase is not used.

Comparative Example 2

The same procedure was conducted as in Example 1 except that Orientase ONS in the step (a) is replaced by Actinase E. The desired product is obtained in an amount of 6.7 mg. Here, peptidase only is used and protease is not used.

CONSIDERATION OF THE RESULTS

From the above, it is seen that the present invention exhibits an excellent effect of two to three times greater yield of the desired product over Comparative Examples 1 and 2 and shows excellent and unexpected effects over the cited references.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed on March 27, 2008.

Kazuhiro Fukae

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